Enzymatic Hydrolysis of Organophosphorus Compounds

VII. The Stereospecificity of Phosphorylphosphatases

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The enzymatic hydrolysis of dimethylamido-ethoxyphosphoryl cyanide (tabun) by purified phosphorylphosphatase preparations from human blood plasma, swine kidney and liver, and by the plasma of rabbit and rat was studied from a kinetic point of view. The hydrolysis was a first-order reaction for the human plasma enzyme. When the kidney and liver enzymes were used, the reactions were composed of two simultaneous first-order reactions; the numerical values of the rate constants for the kidney enzyme were 5.70 h^{-1} and 0.39 h^{-1}. The results were interpreted as being due to stereospecificity for the kidney and liver enzymes, the stereoisomers of tabun being hydrolysed at two different rates by these enzymes. In studies with mixed enzymes it was demonstrated that the isomer of tabun, being slowly hydrolysed by the kidney enzyme, was readily hydrolysed by both the human plasma phosphorylphosphatase and the rabbit plasma enzyme. Both the rat and rabbit plasma phosphorylphosphatases also showed stereospecificity, but to a lower degree compared with the kidney enzyme.

The existence and properties of phosphorylphosphatases have recently been reported by this [1] and other laboratories [2-4]. In most of our studies, tabun (dimethylamido-ethoxyphosphoryl cyanide) was used as substrate. From the reaction curves (see paper I of the present series of reports [1]) for the system tabun-phosphorylphosphatase using various animal tissues as enzyme sources, it can be concluded that the reaction, in certain cases at least, might be composed of two different hydrolysis rates. Hoskin and Trick [5] recently obtained experimental evidence for this proposal and suggested that tabun, containing an asymmetric phosphorus atom, exists in two optically active forms which are enzymatically hydrolysed at different rates. Rat serum was employed in their experiments, and polarimetric determinations indicated that the dextrorotatory form of tabun is more rapidly hydrolysed. Similar experiments have been performed in this laboratory, and extended to a variety of enzyme preparations. The present report deals with the results obtained.
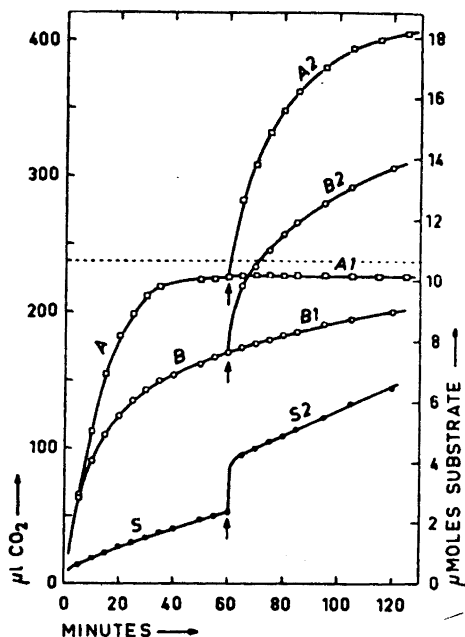
Fig. 1. Enzymatic hydrolysis of tabun by purified phosphorylphosphatase preparations from human serum (A) and swine kidney, and the effects of the addition (indicated by an arrow) of a second portion of enzyme (1) and substrate (2) respectively. S, spontaneous substrate hydrolysis. Dotted line refers to total substrate hydrolysis (273.5 μl CO_2 or 10.6 μmoles).

METHODS AND MATERIAL

Enzyme activity was measured by the Warburg manometric technique at 25°C using flasks with two side bulbs [1]. All determinations were carried out in 0.040 M sodium bicarbonate solution of pH 7.6 (equilibrated with a mixture of 95 % N_2 and 5 % CO_2) and tabun, dissolved in water immediately before use, was used as substrate throughout. The total volume of the reaction mixture was 2.00 ml after adding the first portion of substrate from one of the side bulbs. In most experiments, 1.60 ml of the enzyme solution was placed in the main compartment, and 0.40 ml of the substrate or enzyme solution in each of the two side bulbs. The amount of substrate added to the enzyme from the beginning was 10.6 μmoles of tabun, and this amount was also used when a second portion of substrate was added to the reaction mixture.

Human plasma enzyme was prepared from postpartum serum according to a partly modified method No. 6 of Cohn *et al.*[6]. It was identical with Fraction IV−1 used in previous investigations of this series.

Rabbit plasma enzyme and *rat plasma enzyme.* Fresh plasma was used as such without any treatment.

Kidney and *liver enzymes* were prepared from fresh swine organs, the first part of the method being similar to that described by Mounter *et al.*[2] for DFPase, and continued with ammonium sulphate fractionation according to a procedure to be described in a following report.
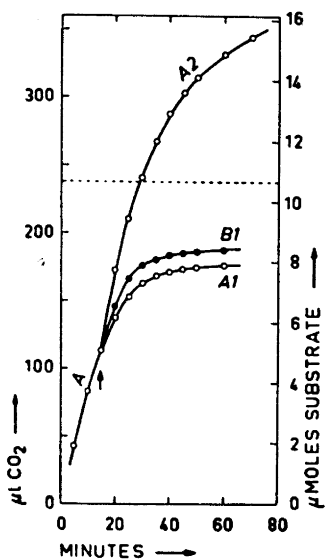
Fig. 2. Enzymatic hydrolysis of tabun by rat plasma (A), and the effects of the addition of a second portion of enzyme (rat plasma, A1; purified kidney enzyme, B1) and substrate (2). *Cf.* Fig. 1.
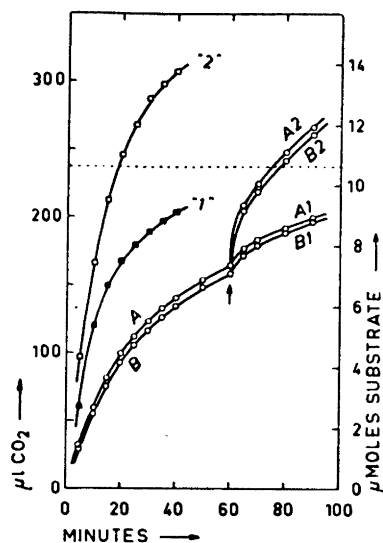
Fig. 3. Enzymatic hydrolysis of tabun by two purified preparations (A and B) of swine liver phosphorylphosphatase, and the effects of the addition of a second portion of enzyme (1) and substrate (2); prepn. A purer than prepn. B. Curves "1" and "2" refer to systems where the concentrations of enzyme and substrate respectively were doubled from the beginning of the reaction. *Cf.* Fig. 1.

RESULTS

Human plasma phosphorylphosphatase. The hydrolysis curve (Fig. 1) showed that tabun was hydrolysed to almost 100 %, suggesting that both the optical isomers of the substrate were split by this preparation. Two identical reaction mixtures were used, and after 60 min when the reaction had come to completion, the same amounts of substrate and enzyme, respectively, as present from the beginning of the experiment were added in order to double the concentration of the two constituents. The addition of more enzyme had no effect, confirming that all the substrate had been used up. After adding the second portion of substrate, on the other hand, the hydrolysis reaction started again with a rate somewhat lower than was observed with untreated enzyme. The lower reaction rate was probably due to enzyme inhibition by the degradation product(s) of tabun.

It will be noted in Fig. 1 and also in the following figures that the theoretical 100 % hydrolysis of the substrate was never reached. This was due to the spontaneous hydrolysis of tabun, *i.e.*, the substrate concentration at the moment of mixing enzyme and substrate is lower than the actual amount of substrate dissolved; it is impossible to avoid such spontaneous hydrolysis

of the substrate solution before use, for practical reasons. The spontaneous hydrolysis curve is inserted in Fig. 1, and was obtained by replacing the enzyme solution with the bicarbonate solution. The acid degradation product of tabun formed (dimethylamido-ethoxy-hydroxy-phosphine oxide) gives rise to an instantaneous production of CO_2, when the substrate solution is mixed with the bicarbonate containing enzyme solution.

Rat plasma phosphorylphosphatase. Rat plasma splits tabun at about the same rate as human plasma. In the experiment shown in Fig. 2 the second portion of substrate and enzyme, respectively, were added before the reaction had come to completion. The rat plasma enzyme seems to split one of the stereoisomers at higher rate than the other isomer. This result confirms the observation reported previously by Hoskin and Trick [3].

With the *rabbit plasma phosphorylphosphatase* similar results were obtained, but the stereospecificity of this enzyme is less pronounced than that of the rat plasma enzyme. This was obvious from an experiment where the rabbit plasma was added to a mixture in which one of the isomers of tabun had been split completely by the kidney phosphorylphosphatase (Fig. 4).

Swine kidney phosphorylphosphatase. The hydrolysis of tabun by purified enzyme of swine kidney (Figs. 1 and 4) is readily interpreted on the basis of enzyme stereospecificity with respect to the substrate. After the gas evolution during the first minutes due to the decomposition of tabun before the addition of the substrate solution to the bicarbonate system, a rapid hydrolysis was observed which came to completion when about one-half (120 μl) of the theoretical yield of gas was evolved. Then the reaction rate became relatively low; this slow reaction was partly due to the spontaneous (non-enzymatic) hydrolysis of the other stereoisomer of tabun and partly to the enzymatic hydrolysis of that isomer. The addition of a second portion of enzyme did not change the reaction rate; after adding a second portion of substrate, on the other hand, the reaction started again with a rate which was lower than following the addition of the first portion of substrate. It is suggested that the phosphorus containing reaction product inhibits the enzymatic hydrolysis, thus explaining the lower reaction rate.

The composition of the reaction curve by two hydrolysis rates are further illustrated in Fig. 5.

Swine liver phosphorylphosphatase. A purified preparation of this enzyme behaved similarly to the kidney enzyme, but the difference between the hydrolysis rates of the two stereoisomers was less pronounced for the former enzyme (Fig. 3).

Studies with mixed enzymes. In order to find confirmation for the suggestion put forward above that human plasma phosphorylphosphatase splits both the stereoisomers of tabun, the following experiment was performed (Fig. 4). The substrate was hydrolysed by the kidney enzyme, and when the enzymatic hydrolysis had come to completion a purified preparation of human plasma enzyme was added to the reaction mixture. It was found that the plasma enzyme split that isomer of the substrate which had not been attacked by the kidney enzyme. A similar result was obtained when the rabbit phosphorylphosphatase was added to the reaction mixture. The same amount
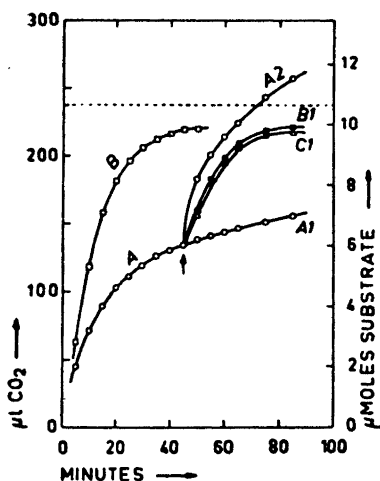
Fig. 4. Enzymatic hydrolysis of tabun by a purified phosphorylphosphatase preparation from swine kidney (A) and the effect of purified phosphorylphosphatase from human serum (B1) and of rabbit plasma (C1) on the reaction mixture. The hydrolysis curve for the purified enzyme from human serum (B) is inserted for comparison. *Cf.* Fig. 1.
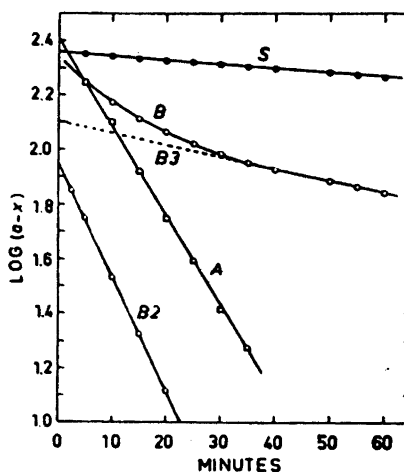
Fig. 5. Graphical evaluation of first-order reaction constants for the enzymatic hydrolysis of tabun by purified phosphorylphosphatase preparations from human serum (A) and swine kidney (B) and the spontaneous (non-enzymatic) hydrolysis of the substrate (S). B3, extrapolated line to estimate k_3 (actually $k_1 + k_3$), and to obtain B2, the line necessary for the estimation of k_2. Experimental values taken from Fig. 1. *Cf.* text.
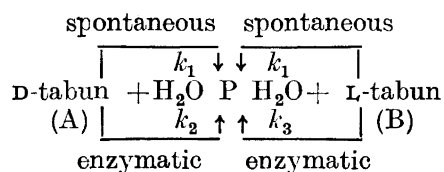
of gas was finally evolved as when the human plasma enzyme hydrolysed the substrate alone from the beginning.

Rat plasma behaved differently in these experiments compared with human and rabbit plasma: it was not found possible to hydrolyse the "non-attacked" isomer. In a similar experiment performed, the result of which is shown in Fig. 2, it was likewise demonstrated that the rat plasma enzyme split the same isomer of tabun as did the kidney enzyme.

DISCUSSION

The results reported above show that the enzymatic hydrolysis of tabun by certain enzyme preparations (especially those from the kidney and liver) is composed of at least two simultaneous reactions. The explanation, is, most probably, that tabun contains an asymmetric phosphorus atom and thus exists in two optically active isomers which are hydrolysed enzymatically at different rates. This idea, first pointed out by Hoskin and Trick [5], is clearly demostrated by a purified enzyme preparation from swine kidney (Fig. 1). The data obtained in this experiment were used to calculate the reaction constants according to a classical graphical technique, and the results are recorded in Fig. 5.

When a racemic mixture of tabun is used as substrate for this particular enzyme (kidney phosphorylphosphatase), the following four first-order reactions are suggested to take place simultaneously (rate constants in brackets): the spontaneous hydrolysis (k_1 which is the same for the two isomers), the enzymatic hydrolysis of the D-isomer (k_2) and the enzymatic hydrolysis of the L-isomer (k_3). Whether the reaction products (P) contain dimethylamido-ethoxy-hydroxy-phosphine oxide in an optically active form or as a racemic mixture has not been investigated:

$$
\begin{array}{cc}
\text{spontaneous} & \text{spontaneous} \\
\hline
k_1 \downarrow\quad \downarrow k_1 & \\
\text{D-tabun} \quad +H_2O \quad P \quad H_2O+ \quad \text{L-tabun} \\
\text{(A)} \quad\quad k_2 \uparrow\quad \uparrow k_3 \quad\quad \text{(B)} \\
\hline
\text{enzymatic} & \text{enzymatic}
\end{array}
$$

The four reactions are represented by the following differential equations:

Spontaneous:

$$-\frac{d(A)}{dt} = k_1(A)$$

$$-\frac{d(B)}{dt} = k_1(B)$$

Enzymatic + spontaneous:

$$-\frac{d(A)}{dt} = k_1(A) + k_2(A)$$

$$-\frac{d(B)}{dt} = k_1(B) + k_3(B)$$

If $\log (a - x)$ (a = original amount of substrate, x = amount of substrate hydrolysed spontaneously and/or enzymatically after t min) is plotted against t, a straight line is obtained for the spontaneous hydrolysis, and from the slope of the line the rate constant, k_1, was found to be 0.21 h^{-1}. The curved line (B) representing the enzymatic hydrolysis by the kidney enzyme (*cf.* Fig. 1, B) indicates that this reaction can be resolved into two first-order reactions, represented by the line B3 (extrapolation of line B to the ordinate) and the line B2 (obtained by subtracting line B3 from B) (Fig. 5). The line B3 in its turn is composed of the spontaneous (k_1) substrate hydrolysis and the enzymatic (k_3) hydrolysis of the slow reacting stereoisomer. The numerical values of k_3 and k_2 obtained were 0.39 h^{-1} (= 0.60 — 0.21) and 5.71 h^{-1}.

The enzymatic hydrolysis of tabun by the purified human plasma phosphorylphosphatase is a first-order reaction, indicated by the straight line obtained when the experimental values from Fig. 1 are analysed according to the same graphical method. The numerical value of the reaction constant in this case was 4.45 h^{-1}. This enzyme, therefore, split both the stereoisomers of tabun at the same rate.

It will be noted that the hydrolysis reaction proceeded in a medium of pH 7.6 at which pH the P-CN is the only linkage broken, both spontaneously [7] and enzymatically [1].

In the experiments reported by Hoskin and Trick [5], using rat serum as the enzyme source, the reaction constant for the spontaneous hydrolysis of tabun was found to be approximately five times greater than that for the slow reaction observed during the enzymatic hydrolysis. It is difficult to

find a plausible explanation for this observation. Such an unexpected difference between the two rate constants has not been found in any of our experiments.

It has not been possible to supplement this investigation by observing changes in optical rotation during the reactions. This is mainly due to technical reasons: a comparatively high tabun concentration is necessary for polarimetric determination, and there are difficulties in getting the enzyme solutions clear enough to make observations of optical rotation possible. Using rat serum, Hoskin and Trick [5] reported some data from polarimetric determinations which indicated that the dextrorotatory isomer of tabun is more rapidly hydrolysed enzymatically. It is likely that this isomer also is the form of tabun which is more readily attacked, for example, by the kidney phosphorylphosphatase.

The results obtained in the present investigation with various enzyme preparations support the idea that there exists a variety of phosphorylphosphatases. The enzyme present in human plasma differs from those present in the kidney and liver by not showing stereospecificity. Furthermore, the enzymes of rat and rabbit plasmas behave differently in this respect both from the human plasma enzyme and the enzymes present in the organs studied. In a report to be published in due course, further support for the complexity of phosphorylphosphatases will be discussed.

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